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ORIGINAL PAPER

Non-mycorrhizal fungal endophytes in two orchids of Kaiga forest (Western Ghats), India

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Abstract: We used standard isolation protocols to explore the endophytic fungal communities in three tissue types of two dominant orchids (*Bulbophyllum neilgherrense* and *Vanda testacea*) of the Kaiga forest of the Western Ghats. We surface sterilized and assessed 90 segments of each orchid for the occurrence and diversity of endophytic fungal taxa. The 118 fungal isolates were obtained from root, bulb and leaves of *B. neilgherrense*, consisting of 17 anamorphic taxa (range, 10–15 taxa) with 1.3 fungal taxa per segment (range, 1.2–1.4 taxa). Four taxa (*Aspergillus flavus, A. niger, Penicillium* sp. and morpho sp. 1) belonged to the core group (11.1%–32.2%). The relative abundance of *A. flavus* and morpho sp. 1 was more than 10%. A total of 130 fungal isolates from roots, stems and leaves of *V. testacea* yielded 20 anamorphic taxa (range, 11–15 taxa) with 1.4 fungal taxa per segment (range, 1.4–1.5 taxa). *Aspergillus flavus, A. niger, A. ochraceus*, *Gliocladium viride, Penicillium* sp. and morpho sp. 1 belonged to the core group. Relative abundance exceeded 10% for *A. flavus, A. niger*, and morpho sp. 1. The Simpson and Shannon diversity indices were higher in leaf than root or bulb/stem of both orchids. Jaccard's similarity coefficient was higher between root and leaf in both orchids (56.3%–60%) than between other pairs. Our study revealed that the endophytic fungal assemblage and diversity of *B. neilgherrense* and *V. testacea* of Kaiga forest of the Western Ghats were relatively similar between orchids and their tissues.

Keywords: orchids; *Bulbophyllum*; *Vanda*; endophytic fungi; Western Ghats

Introduction

Endophytes belong to a wide range of organisms (bacteria and fungi) inhabiting the healthy plant tissues without causing visible pathological symptoms (Peters 1991; Petrini 1991; Kobayashi et al. 2000; Stone et al. 2000; Schulz et al. 2005; Hyde and Soytong 2008). Various definitions of endophytes and their importance were reviewed by Hyde and Soytong (2008). Endophytic fungi are polyphyletic, functionally diverse, and serve as latent pathogens, mutualists (mycorrhizas) or saprophytes involved in decomposition, nutrient turnover, anti-herbivory and symbiotic benefits (Christensen 1989; Gardes 2002; Sieber 2002; Schulz and Boyle 2005; Vega et al. 2008). Endophytic fungal associations increase host plant fitness to abiotic stresses (Redman et al. 2002; Bae et al. 2008) and improve plant adaptability to various environmental conditions (Bonnardeaux et al. 2007; Swarts et al. 2010). Fungi occurring at ≥10% frequency are referred to as 'core group fungi' (Alias et al. 1995; Sarma et al. 2001) and such dominant endophytic fungi may play a major role in plant fitness. The endophytic fungal guild (transmitted vertically or horizontally) in plants serves as an important source of unknown and cryptic fungi as only about 7% of the estimated 1.5×10^6 fungal taxa are known (Hawksworth 2004). Endophytic fungi are metabolically versatile and serve as a source of novel natural products of pharmaceutical and agricultural value (Tan et al. 2001; Gunatilaka 2006; Schulz et al. 2002; Tao et al. 2008; Suryanarayanan et al. 2009).

Even though Orchidaceae is a large family encompassing 10% of flowering plants (Jones 2006), investigations on symbiotic association of terrestrial orchids have focused mainly on mycorrhizal fungi rather than non-mycorrhizal fungi (Shefferson et al. 2005; Bonnardeaux et al. 2007; Irwin et al. 2007; Kaliamoorthy 2007; Zhi-lin Yuan et al. 2009; Bagyalakshmi et al. 2010). Several anamorph and teleomorph fungal taxa are known as mycorrhizal associates of orchids (for example, anamorph: *Ceratorhiza*, *Epulorhiza* and *Moniliopsis*; teleomorph: *Ceratobasidium*, *Guignardia*, *Oliveonia* and *Rhizoctonia*), (Pereira et al. 2003, 2005; Taylor et al. 2003; Zettler et al. 2004; Tao et al. 2008; Rungjindamai et al. 2008; Zhi-lin Yuan et al. 2009; Pinuran et al. 2010). Although orchids host many non-mycorrhizal fungi in leaves, studies on such fungi especially in tropical and subtropical orchids are lacking (Rasmussen 2002; McCormick et al. 2004;

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Bayman et al. 2006; Suarez et al. 2006; Dearnaley 2007; Zhi-lin Yuan et al. 2009). Few studies have been carried out on the mycorrhizal colonization of orchids of the Western Ghats (Kaliamoorthy 2007; Bagyalakshmi et al. 2010) and the fungal endophytes of orchids are mostly known. The aim of the current study was to assess the assemblage and occurrence of endophytic fungi in root, stem and leaf tissues of two dominant orchids of the Kaiga forest of the Western Ghats of Southwest India.

Materials and methods

Sampling site and orchids

The Western Ghats mountain range (N 8º20'–20º40' and E 73º–77º) stretching about 1,600 km (140,000 km^2) along the west coast of India, is a hotspot of biodiversity. Grasslands, shoals, moist-dry deciduous forests, evergreen forests and scrub jungles are the main forest vegetation types distributed in different altitudes of the Western Ghats. Among 5,000 flowering plant taxa in the Western Ghats, about one-third (1,720 taxa of 54 genera) are endemic. Orchids, consisting of about 310 taxa (75 genera, 4 subspecies and 3 varieties), represent approximately 40% of the taxa endemic to the Western Ghat forests (Abraham et al. 1981; Kumar et al. 2001). Trees of the Western Ghats harbor a variety of epiphytic orchids (Kumar et al. 2001). The sampling location (Kaiga forest) is situated adjacent to the River Kali (35 km east of Karwar; at 55−70 m a.s.l.) of southwest India and receives average rainfall of about 280 cm per year. This forest is dominated by two epiphytic orchids viz., *Bulbophyllum neilgherrense* Wight and *Vanda testacea* (Lindl.) Reichb. f. (syn: *Vanda parviflora* Lindl.). We collected five healthy plants of each orchid during November 2009 (post-monsoon season) from the tree canopies of *Artocarpus heterophyllus* Lam., *Ficus benghalensis* Linn. and *Syzygium caryophyllatum* (L.) Alston., and transported them to the laboratory in air-tight polyethylene bags. Orchid tissues were processed and plated on media within 24 h of sampling for isolation of endophytic fungi.

Fungal isolation

The plants were gently rinsed in freshwater to remove extraneous matter. From each plant, six segments (4-5 mm × 30 mm) of mature aerial roots, mature bulb (*Bulbophyllum*) / stem (*Vanda*) and mature leaves were excised. These 30 segments of each tissue per orchid were surface-sterilized according to Taylor et al. (1999) with a slight modification. Each set of plant segments was immersed in 95% ethanol for 1 min, followed by immersion in 0.6% sodium hypochlorite (British Drug House, E. Merck India Ltd., Mumbai, India) for 5 min and then immersed in 95% ethanol for 0.5 min. The sterilized segments were immediately rinsed thrice in sterile distilled water before plating onto 1.5% malt extract agar (MEA) medium supplemented with terramycin (250 mg· L^{-1}), (Sigma Chemical Co., St. Louis, Missouri). The plates were incubated at (25 ± 2) °C under a fluorescent light (intensity 0.1 mW) with a 12-hour photoperiod in the laboratory for 6–8 weeks. Growing regions of fungi from tissues were subcultured for identification on fresh antibiotic-free MEA medium. Non-sporulating morphospecies obtained on 1.5% MEA medium were subcultured on potato dextrose agar (PDA) medium (HiMedia Laboratories Pvt. Ltd., Mumbai, India) to induce sporulation. Mycelia of each fungus were mounted with cotton blue in lactophenol to observe spore morphology and ontology for identification. Endophytic fungi recovered from the orchids were identified, based on the monographs written by Ellis (1971, 1976), Carmichael et al. (1980), Ellis and Ellis (1987), Cai et al. (2006) and Bhat (2010).

Data analysis

We estimated colonization frequency (%) and relative abundance (%) of each fungus on orchid segments:

$$C_F = (N_s \div T_s) \times 100 \quad (1)$$

$$R_a = (F_o \div T_o) \times 100 \quad (2)$$

where, C_F is the colonization frequency (%), N_s the number of segments colonized, T_s the total segments examined; R_a the relative abundance (%), F_o the frequency of occurrence of specific fungus, and T_o is the total frequency of occurrence of all fungi.

We calculated diversity (D', Simpson; H', Shannon) (Magurran 1988) and evenness (J', Pielou's), (Pielou 1975) of fungal taxa on each tissue and for all tissues:

$$D' = 1 \div \sum (p_i)^2 \quad (3)$$

$$H' = -\sum (p_i \ln p_i) \quad (4)$$

where, p_i is the proportion of the ith taxon.

$$J' = (H' \div H'_{\max}) \quad (5)$$

where, H'_{max} is ln$S$; S is total number of species in the community.

To compare the richness of fungal taxa in orchids, numbers of isolations and numbers of segments were assessed (tissue-wise and in all tissues). The expected number of taxa was calculated, based on rarefaction indices (Ludwig and Reynolds 1988). The expected number of fungal taxa, $E_{(t)}$, in a random sample of n fungal isolations taken from a total population of N fungal isolations was estimated:

$$E_{(t)} = \sum_{i=1}^{s} \left\{ 1 - \left[\binom{N - n_i}{n} \Big/ \binom{N}{n} \right] \right\} \quad (6)$$

where, n_i is the number of fungal isolations of the ith taxon.

Jaccard's index of similarity (J_I) in percent was estimated

among the tissues of orchids, based on the presence or absence of each fungal taxon (Kenkel and Booth 1992):

$$J_I(\%) = (c \div a + b + c) \times 100 \quad (7)$$

where, c is the number of fungal taxa common to both tissues, a is the number of fungal taxa unique to the first tissue, and b is the number of fungal taxa unique to the second tissue.

Results

Fungal growth began after one week of incubation of orchid tissues, indicating a successful surface sterilization. Of 90 assessed tissue segments (root, bulb and leaf), 95.6% of orchid tissues yielded 118 endophytic fungal isolates in *Bulbophyllum* (Table 1). Total isolates were higher in bulbs than in root and leaf segments (42 vs. 36-40). The diversity was higher in leaves than in other tissues. A total of 17 anamorphic taxa were recovered with a maximum of 15 taxa in roots (Table 2). The relative abundance of *Aspergillus flavus* and morpho sp. 1 was above 10% (17.1%-24.8%). Overall, four taxa (*A. flavus, A. niger, Penicillium* sp. and morpho sp. 1) were considered core group fungi (11.1%-32.2%). However, *Aspergillus ochraceus, Aspergillus* sp. 1, *Penicillium chrysogenum* and morpho sp. 2 (in roots), *Aspergillus tamari* (in leaves), *Gliocladium viride* (in bulbs) (Fig. 1A) were core group fungi. The fungal similarity among tissues was highest between root and leaf tissues (60%), (Table 3). *Fusarium* sp. isolated from the root segments did not sporulate on MEA medium, but sporulation was induced upon subculturing in PDA medium. However, three morphospecies did not sporulate on the MEA or PDA media.

In *Vanda*, 90 tissue segments yielded a total of 130 isolates (Table 1). Total isolates were slightly higher in roots than in stems or leaves (45 vs. 42-43). Diversity was higher in leaves than that in roots or stems. Three tissues of *Vanda* yielded 20 anamorphic taxa with a maximum of 15 taxa in leaves (Table 2). The relative abundance of *Aspergillus flavus, A. niger* and morpho sp. 1 was above 10% (12.5%-24.1%). Altogether six taxa viz., *Aspergillus flavus*, *A. niger*, *A. ochraceus*, *Gliocladium viride*, *Penicillium* sp. and morpho sp. 1 (Fig. 1B) were assigned to the core group (10%-34.4%). However, *Nigrospora* sp. (in roots) and *Aspergillus candidus* (in stems) were also core group taxa. Fungal similarity among tissues was highest between root and leaf tissues (56.3%), (Table 3). Two morphospecies did not sporulate on the MEA or PDA media, indicating the necessity to use different techniques to induce sporulation. In *Fusarium* sp. in *Bulbophyllum*, *Fusarium oxysporum*, leaf segments of *Vanda* did not sporulate on MEA medium, but did on PDA medium.

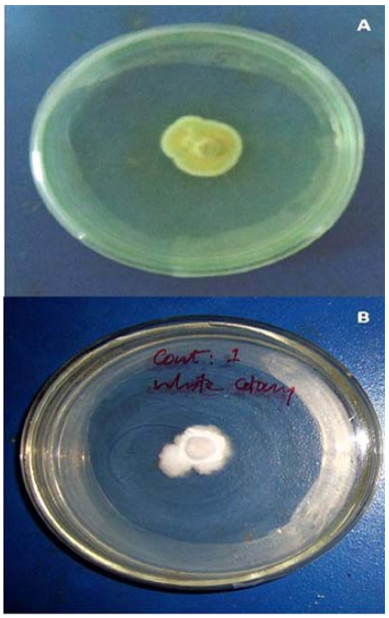

Fig. 1 *Gliocladium viride* isolated from the bulb segment of *Bulbophyllum neilgherrense* grown on malt extract agar medium (A) and morpho species 1 isolated from the stem segment of *Vanda testacea* grown on potato dextrose agar medium (B).

Table 1. Fungal assemblage, species richness, and diversity indices of endophytic fungi of two epiphytic orchids (*Bulbophyllum neilgherrense* and *Vanda testacea*) of Kaiga forest

Orchid tissue	Assemblage*				Species richness**		Diversity		
	Sa	Sco	Fi	Fts	Total taxa	$E_{(t25)}$	Simpson index	Shannon- Weaver Index	Pielou's evenness
Bulbophyllum neilgherrense									
Root	30	28	40	1.3	15	20	0.478	1.839	0.471
Bulb	30	29	42	1.4	10	20	0.828	2.882	0.868
Leaf	30	29	36	1.2	12	21	0.839	3.079	0.859
Overall	90	86	118	1.3	17	23	0.876	3.460	0.846
Vanda testacea									
Root	30	30	45	1.5	11	20	0.863	3.109	0.899
Stem	30	30	42	1.4	13	21	0.848	3.173	0.858
Leaf	30	30	43	1.4	15	20	0.891	3.500	0.896
Overall	90	90	130	1.4	20	23	0.880	3.546	0.826

Notes: *Assemblage: Sa is Number of segments assessed; Sco is Number of segments colonized; Fi is Total fungal isolations; Fts is Number of fungal taxa per segment; **Species richness: $E_{(t25)}$, Expected number of fungal taxa out of 25 random isolations.

Table 2. Colonization frequency (C_F%) of fungal taxa on root (1), bulb/stem (2), and leaf (3) segments of *Bulbophyllum neilgherrense* and *Vanda testacea* of Kaiga forest (out of 30 segments of each tissue)

Taxon	C_F (%)			TFO (%)	R_a (%)
	Root	Bulb	Leaf		
Bulbophyllum neilgherense					
*Morpho sp. 1	30	43.3	23.3	32.2	24.8
**Aspergillus flavus* Link	10	20	36.7	22.2	17.1
**Aspergillus niger* Tiegh.	6.7	20	10	12.2	9.4
**Penicillium* sp.	16.7	10	6.7	11.1	8.5
**Aspergillus ochraceus* G. Wilh.	10	6.7	3.3	6.7	5.2
**Aspergillus tamari* Kita	6.7	6.7	10	7.8	6
**Gliocladium viride* Matr.	6.7	16.7	–	7.8	6
Aspergillus sp. 1	10	–	6.7	5.6	4.3
Morpho sp. 2	10	–	6.7	5.6	4.3
**Penicillium chrysogenum* Thom	10	3.3	–	4.4	3.4
**Aspergillus oryzae* (Ahlb.) E. Cohn	3.3	–	6.7	3.3	2.5
**Penicillium italicum* Wehmer	3.3	6.7	–	3.3	2.5
Aspergillus sp. 2	–	3.3	3.3	2.2	1.7
Morpho sp. 3	3.3	–	3.3	2.2	1.7
**Aspergillus carbonarius* (Bainier) Thom	–	–	3.3	1.1	0.8
Fusarium sp.	3.3	–	–	1.1	0.8
Nigrospora sp.	3.3	–	–	1.1	0.8
***V**anda testacea*	Root	Stem	Leaf		
*Morpho sp. 1	33.3	43.3	26.7	34.4	24.1
**Aspergillus niger* Tiegh.	26.7	20	16.7	21.1	14.8
**Aspergillus flavus* Link	23.3	10	20	17.8	12.5
**Gliocladium viride* Matr.	10	6.7	20	12.2	8.5
**Penicillium* sp.	16.7	13.3	6.7	12.2	8.5
**Aspergillus ochraceus G. Wilh.*	6.7	13.3	10	10	7
Nigrospora oryzae (Berk. & Broome) Petch	6.7	–	6.7	4.4	3.1
Nigrospora sp.	13.3	–	–	4.4	3.1
Aspergillus candidus Link	–	10	–	3.3	2.3
**Aspergillus carbonarius* (Bainier) Thom	–	6.7	3.3	3.3	2.3
**Aspergillus oryzae* (Ahlb.) E. Cohn	–	3.3	6.7	3.3	2.3
**Penicillium chrysogenum* Thom	6.7	–	3.3	3.3	2.3
**Penicillium italicum* Wehmer	3.3	–	6.7	3.3	2.3
Gliocladium sp.	–	3.3	3.3	2.2	1.5
Morpho sp. 4	3.3	–	3.3	2.2	1.5
Alternaria sp.	–	3.3	–	1.1	0.8
**Aspergillus tamari* Kita	–	3.3	–	1.1	0.8
Fusarium oxysporum E.F. Sm. & Swingle	–	–	3.3	1.1	0.8
Paeciliomyces sp.	–	3.3	–	1.1	0.8
Phialophora sp.	–	–	3.3	1.1	0.8

Notes: TFO% is total percent frequency of occurrence out of 90 segments; R_a% is percent relative abundance; *common to both orchids.

Overall 11 fungal taxa were common to *Bulbophyllum* and *Vanda* (Table 2). A comparison of fungal taxa in different tissues of orchids is given in Fig. 2 A, B. In *Bulbophyllum*, *Fusarium* sp. and *Nigorspora* sp. were restricted to roots, while *Aspergillus carbonarious* was restricted to leaves and no taxa were restricted only to bulbs. The number of taxa common to three tissues was higher than for any pair of tissues (6 vs. 1-4) and four of them belonged to core group fungi (Table 2). In *Vanda*, *Nigrospora* sp. was confined to roots, while four and two taxa were confined to stems and leaves, respectively. Common taxa to three tissues were higher (6 vs. 0-4) and all six assigned to the core group (Table 2, 3). The species abundance curve showed that fungal taxa in *Vanda* had higher frequency of occurrence than in *Bulbophyllum* (Fig. 3). Upon comparison of three tissue types of

orchids by rarefaction curves, the expected number of taxa, $E_{(t)}$, showed steep elevation (Fig. 4, Table 1).

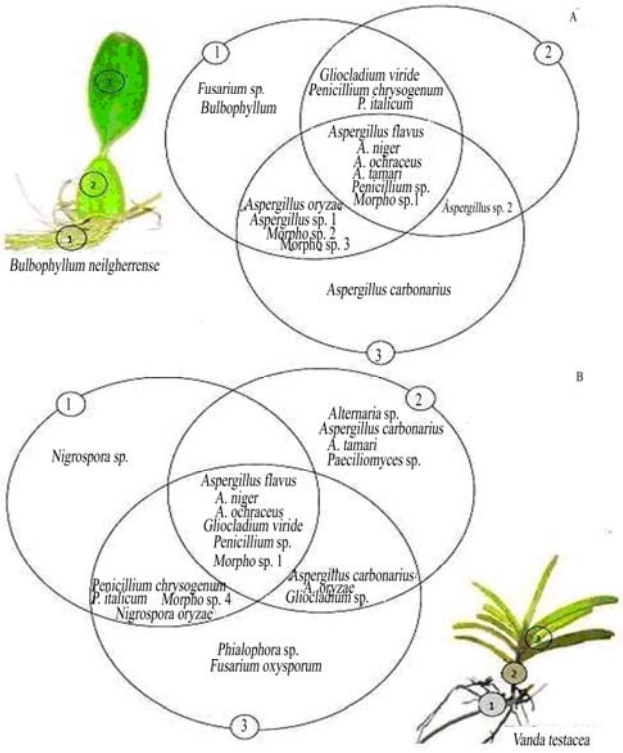

Fig. 2 Comparison of common endophytic fungal taxa in different tissue (1 is root; 2 is bulb/stem; 3 is leaf) segments of *Bulbophyllum neilgherrense* (A) and *Vanda testacea* (B) in Kaiga forest

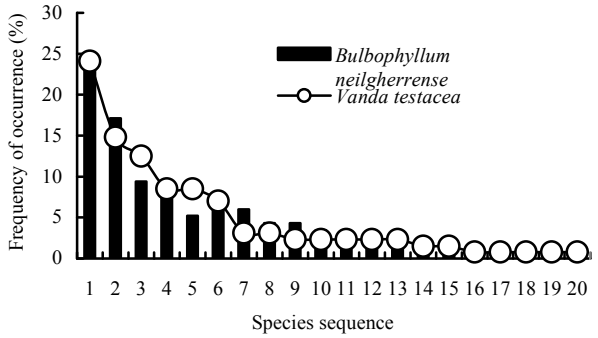

Fig. 3 Species abundance curves of endophytic fungal taxa associated with two orchids (*Bulbophyllum neilgherrense* and *Vanda testacea*) of Kaiga forest (arranged in descending order)

Table 3. Jaccard's similarity coefficient (%) of endophytic fungi in different tissues of *Bulbophyllum neilgherrense* and *Vanda testacea* (in brackets) of Kaiga forest

Tissue	Root	Bulb (Stem)	Leaf
Root	100	56.3 (33.3)	60 (56.3)
Bulb/Stem	-	100	46.7 (52.6)
Leaf	-	-	100

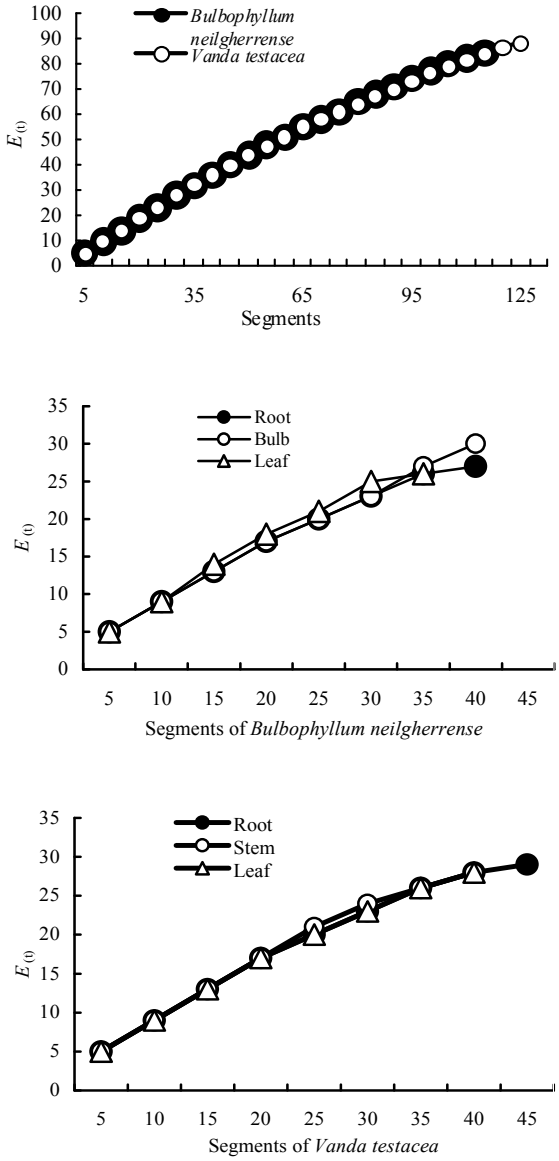

Fig. 4 Rarefaction curve of fungal taxa in tissues of *Bulbophyllum neilgherrence* and *Vanda testacea* in Kaiga forest (number of segments vs. expected number of taxa)

Discussion

Orchids are dependent on fungi for nutrition, growth, and development after seed germination (Smith and Read 1997; Bonnardeaux et al. 2007). Besides mycorrhizal fungi, the non-mycorrhizal fungi of orchids are important due to their multiple ecological roles (Bayman and Otero 2006; Zhi-lin Yuan et al. 2009). Few studies are available on endophytic fungal taxa of different tissues of tropical orchids (Bayman et al. 1997; Zhi-lin Yuan et al. 2009). Among the roots and leaves of five taxa of epiphytic and lithophytic orchids of the genus *Lepanthes* of the Caribbean and Puerto Rico, endophytic fungal taxa as well as isolates were considerably more numerous in roots (Bayman et al.

1997). The species richness index of endophytic fungi was higher in stems than that in roots or leaves of *Dendrobium* in Southwest China (Zhi-lin Yuan et al. 2009). Recently, mycorrhizal and endophytic fungi of roots and seeds of *Dendrobium* sp. orchids have been studied with reference to their importance in antimicrobial activities, production of secondary metabolites and growth-promoting potential (Chen and Guo 2005; Chen et al. 2010, 2012).

Tropical forests have been considered hotspots of foliar endophytic fungi (Arnold and Lutzoni 2007b). Endophytes inhabiting leaves (short-lived, photosynthetically versatile, and subject to damage by herbivores) are under high selective pressure, compared to those fungi associated with persistent tissues (bark, xylem or other woody parts) (Arnold 2007a). Our study revealed higher species richness in roots of *Bulbophyllum* and leaves of *Vanda*. It is likely that leaves of orchids possess diverse endophytic fungal taxa of ecological and economical values. Up to 10 species of *Bulbophyllum* and four species of *Vanda* are widely distributed in the forests of Western Ghats (Ganesan and Livingstone 2001; Kumar et al. 2001; Swamy et al. 2004). Among 1,720 taxa of 54 genera of endemic flowering plants in the Western Ghats, nearly one-third are threatened and some believed to be extinct or at serious risk of extinction (Kaveriappa and Shetty 2001). Thus, several epiphytic orchids are an invaluable source of mycorrhizal and non-mycorrhizal endophytic fungi. Besides several anamorphs and teleomorphs, orchids are also known to harbor some of the aquatic hyphomycetes as endophytes. Molecular analysis of Internal Transcribed Sequences (ITS) revealed the occurrence of sequences *Tetracladium* in roots of an endangered orchid *Orchis militaris* in Northern Italy (Elena et al. 2010). Similarly, the ITS sequences of *Tetracladium* were also found in roots of *Cephalanthera longifolia* of Estonia (Abadie et al. 2006) and *Gymnadenia conopsea* in East Germany (Stark et al. 2009). Many species of *Tetracladium* have been linked as aquatic, endophytic, or soil inhabitants by Selosse et al. (2008). Besides *Tetracladium*, several aquatic hyphomycetes (*Dwayaangam colodena, Tripospermum camelopardus* and *T. myrti*) are endophytic in black spruce needles (*Picea mariana*) in a mixed wood forest canopy in Canada (Sokolski et al. 2006). In the present study, no aquatic hyphomycetes were found upon culturing on MEA and PDA media. However, production of conidia of aquatic hyphomycetes cannot be ruled out (Sridhar and Bärlocher 1992; Sokolski et al. 2006).

We encountered co-occurrence of many fungal taxa in the orchid tissues seen in our study. Although 2-3 taxa of fungi grew in close proximity on the tissue segments, they did not inhibit each other and they also did not intermingle their hyphae. Occurrence of potential toxin producing fungi such as *Aspergillus flavus*, *A. ochraceus* (in both orchids) and *Gliocladium viride* (in *B. neilgherrense*) at high frequency seems to protect orchids from herbivores. Likewise, many entomopathogenic fungi have been reported as endophytes in several plant species (Petrini 1981; Bills and Polishook 1991; Ananda and Sridhar 2002; Seena and Sridhar 2004; Vega et al. 2008). For instance, *Paeciliomyces* sp. in stems of *Vanda* in our study may serve as potential entomopathogenic fungi. Antimicrobial activity of orchid endophytic fungi was reported by Aline Vaz et al. (2009) and Chen et al. (2010). Our preliminary study on *Gliocladium viride* and morpho sp. 1 based on the cross-streak method showed inhibition against *Bacillus subtilis*, *Escherichia coli*, and *Candida albicans*, providing a basis for further investigation on different endophytic fungi of orchids.

The overall composition of endophytic fungal taxa in our study was more or less similar in both orchids as 11 taxa were common with four core group taxa. The expected number of fungal taxa showed exponential elevation with increased segments of both orchids, suggesting more diverse fungal taxa could be recorded upon screening a higher number of tissue segments. Roles of non-mycorrhizal endophytic fungi in orchids need further investigation. In addition to investigation of roots, stems, and leaves, future studies on orchid endophytes need to evaluate seeds and inflorescences, which might be helpful to understand the role of endophytic fungi in orchid biology, cultivation, conservation, and natural product recovery.

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